The contribution of VHL substrate binding and HIF1-α to the phenotype of VHL loss in renal cell carcinoma

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Summary

Clear-cell renal carcinoma is associated with inactivation of the von Hippel-Lindau (*VHL*) tumor suppressor gene. VHL is the substrate recognition subunit of an E3 ligase, known to target the α subunits of the HIF heterodimeric transcription factor for ubiquitin-mediated degradation under normoxic conditions. We demonstrate that competitive inhibition of the VHL substrate recognition site with a peptide derived from the oxygen degradation domain of HIF1α recapitulates the tumorigenic phenotype of VHL-deficient tumor cells. These studies prove that VHL substrate recognition is essential to the tumor suppressor function of VHL. We further demonstrate that normoxic stabilization of HIF1α alone, while capable of mimicking some aspects of VHL loss, is not sufficient to reproduce tumorigenesis, indicating that it is not the critical oncogenic substrate of VHL.

Introduction

von Hippel-Lindau (VHL) disease is an autosomal dominant cancer syndrome caused by inactivation of the *VHL* tumor suppressor gene by mutation, deletion, or hypermethylation. Affected individuals are at risk to develop highly vascular tumors in a number of organs, including brain, adrenal, and pancreas, as well as clear cell tumors of the kidney (Choyke et al., 1995). It is now clear that 50%–80% of sporadic clear cell renal cell carcinomas also demonstrate biallelic loss of *VHL* (Gnarra et al., 1994; Foster et al., 1994; Herman et al., 1994), suggesting a common VHL tumorigenic pathway for the majority of renal carcinoma.

Until recently, very little was known about the function of VHL. Examination of VHL-deficient renal cell carcinoma cell lines reveals multiple phenotypes that are abrogated by reintroduction of wild-type VHL. Biochemically, deficient cells demonstrate increased transcription of a number of genes, including vascular endothelial growth factor (VEGF) (Siemeister et al., 1996) and glucose transporter-1 (Glut-1) (Iliopoulos et al., 1996). Deficient cells have a disrupted extracellular matrix due to inappropriate processing of fibrinogen (Ohh et al., 1998) and undergo abnormal growth in response to serum starvation (Pause et al., 1998). When grown in a collagen matrix, deficient cells branch and migrate in response to hepatocyte growth factor/scatter factor (HGF/SF) (Koochekpour et al., 1999). Finally, VHL deficient cells form tumors when implanted in immunocompromised mice (Iliopoulos et al., 1995).

It is now clear that VHL is the substrate recognition component of an E3 ubiquitin ligase complex (Duan et al., 1995; Kibel et al., 1995; Pause et al., 1997, 1999; Kamura et al., 1999) that targets hypoxia inducible factors 1α (HIF1α) and 2α (HIF2α) for ubiquitin-mediated degradation under normoxic conditions (Cockman et al., 2000; Ohh et al., 2000). HIF1α and HIF2α are α subunit isoforms of a heterodimeric transcription factor responsible for hypoxia-dependent regulation of a number of genes associated with angiogenesis and erythropoeisis, including VEGF (Forsythe et al., 1996), Glut-1, PDGFα, and erythropoeitin (Bunn et al., 1998; Semenza and Wang, 1992). Although HIF1α and HIF2α share only 48% identity, they both contain a conserved 15 amino acid minimal VHL binding domain, and both bind and activate the same DNA recognition element (Tian et al., 1997; Flamme et al., 1997). Under normoxic conditions, key proline residues of HIFα are hydroxylated by a recently described family of oxygen-dependent prolyl hydroxylases (Epstein et al., 2001). Unhydroxylated HIFα does not bind VHL, and thus accumulates in the cell (Jaakkola et al., 2001; Ivan et al., 2001). All renal tumor-causing VHL mutants thus far exam-

SIGNIFICANCE

Loss or inactivation of the von Hippel-Lindau (*VHL*) tumor suppressor gene is implicated in more than 60% of sporadic clear cell renal cell carcinoma cases. VHL forms the recognitions subunit of an E3 ubiquitin ligase, which targets hypoxia inducible factor α subunit isoforms, HIF1α and HIF2α, for ubiquitin-mediated degradation. VHL associated renal cell tumors demonstrate defective ubiquitination of HIF subunits, suggesting that this function is critical to tumor suppression by VHL. To test this, we blocked binding of VHL to HIF subunits in renal tumor cells reexpressing VHL and demonstrated recovery of tumor growth in SCID mice, indicating a crucial role for HIF regulation in tumorigenesis in these cells.

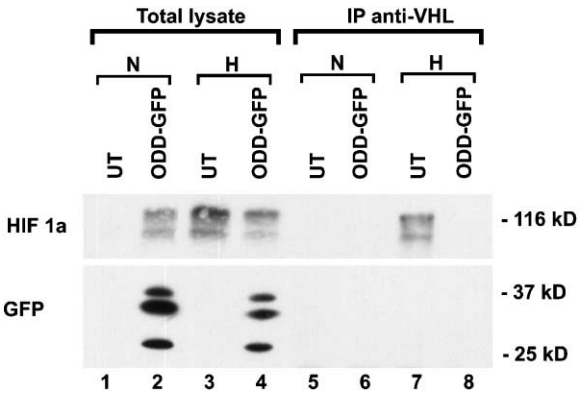

Figure 1. Induction of HIF1α by ODD-GFP in HeLa cells

A representative immunoblot demonstrating induction of HIF1α protein in untransfected (UT) cells by exposure to 8 hr of 0.5% O_2 (H), and similar induction in normoxia (N) when the ODD-GFP fusion was present. Corresponding anti-VHL immunoprecipitation (IP) reveals loss of VHL-HIF1α co-IP when ODD-GFP was expressed.

ined result in defective ubiquitination of HIFα (Cockman et al., 2000; Ohh et al., 2000). Although HIF1α and HIF2α are the only documented substrates of the VHL E3 ubiquitin ligase, others may exist.

Although stabilization of HIFα is consistent with the elevated VEGF levels and increased vascularity characteristic of VHL-associated tumors, it is unclear whether all aspects of the phenotype associated with VHL loss and reversible by VHL reintroduction can be directly attributed to substrate degradation by VHL. To address this, we competitively inhibited VHL-substrate binding using a peptide derived from the VHL binding domain of HIF1α. Expression of the peptide fully recapitulated the tumorigenic phenotype of VHL negative cells, indicating that VHL-substrate recognition is critical for tumor suppression by VHL. We further demonstrate that expression of a HIF1α variant that is unable to bind VHL mimics some aspects of VHL loss but is not tumorigenic, suggesting that HIF1α is not the critical substrate of VHL for tumor formation.

Results

To competitively inhibit HIF1α binding and degradation by VHL, we created a recombinant fusion construct containing the oxygen-dependent degradation domain (ODD) of HIF1α followed by green fluorescent protein (GFP), ODD-GFP. We initially tested the effect of the construct on HIF1α expression in HeLa cells for ease and efficiency of transfection (Figure 1). Untransfected HeLa cells had no detectable HIF1α in normoxia (lane 1) but demonstrated HIF1α induction when grown in 0.5% O2 for 8 hr (lane 3). Transient transfection with ODD-GFP stabilized endogenous HIF1α protein to levels approaching those seen in hypoxia (lane 2), suggesting that the fusion was effectively preventing HIF1α degradation. When ODD-GFP transfected cells were grown in hypoxia, no further induction of HIF1α was seen (lane 4), suggesting that hypoxia was not contributing to HIF1α induction by alternate pathways. The same four lysates were immunoprecipitated with anti-VHL Ab to assess binding of VHL to HIF1α or ODD-GFP (lanes 5–8, respectively). Lane 7 demonstrated coimmunoprecipitation of HIF1α and VHL, which was expected on reoxygenation of the lysate (Cockman et al., 2000). In contrast, interaction between HIF1α and VHL was not detected in lysate prepared from the ODD-GFP transfectant, despite high levels of HIF1α. This observation is consistent with successful competition for VHL binding by ODD-GFP. We were unable to detect coimmunoprecipitation of ODD-GFP with endogenous VHL from these lysates. To improve detection of binding between VHL and ODD-GFP, we cotransfected cells with wild-type VHL. The addition of exogenous VHL enabled demonstration of ODD-GFP coimmunoprecipitation with VHL (data not shown) confirming direct binding.

To evaluate transcription of HIF1α target genes, ODD-GFP was cotransfected with luciferase reporter constructs containing the minimal hypoxia responsive promoter of either erythropoietin (Figure 2A) or VEGF (Figure 2B). Untransfected HeLa cells demonstrated low levels of transcription of both reporters in normoxia with 5- to 10-fold increase of luminescence in hypoxia. Normoxic transcription of both reporters approached hypoxic levels when cells were transfected with ODD-GFP but not with GFP alone, demonstrating that the nondegraded endogenous HIF1α was transcriptionally active in normoxia. ODD-GFP transfected cells grown in hypoxia did not show any further increase in VEGF reporter transcription, suggesting that the full hypoxic effect was duplicated by the fusion construct.

Although we had shown that ODD-GFP stabilized HIF1α protein and induced transcription of hypoxia-responsive genes by blocking the interaction between HIF1α and VHL, we could not rule out the possibility of alternative mechanisms for these effects, including blockade of an interaction between VHL and another substrate. To specifically address the role of HIF1α protein level regulation in the phenotypes associated with the expression or loss of VHL, we next created a HIF1α mutant that was unable to bind VHL. Site-directed mutagenesis was used to substitute five amino acids of HIF1α within the conserved VHL binding domain including proline 564, the target of the HIF prolyl hydroxylase (Epstein et al., 2001; Ivan et al., 2001). We first introduced this mutation into the ODD-GFP fusion construct to confirm that it eliminated VHL binding. The mutation, ODD-GFP(M2), abrogated competitive inhibition of endogenous HIF1α degradation (data not shown). Consistent with this finding, VEGF-luciferase reporter activity was not induced above normoxic background levels by the mutant ODD-GFP(M2) (Figure 2C). Next, we introduced the same mutation into a full-length HIF1α cDNA, HIF1α(M2). HeLa cells cotransfected with wild-type HIF1α and the VEGF-luciferase reporter demonstrated some transcriptional activation, possibly attributable to overexpression of HIF1α beyond the capacity of endogenous VHL to degrade. Cotransfection with HIF1α(M2), however, induced reporter activity approaching the high levels seen in hypoxia. This demonstrated that although the mutant HIF1α had lost the ability to bind VHL, it remained transcriptionally active (Figure 2D).

We now had two constructs, ODD-GFP and HIF1α(M2), capable of abrogating oxygen-dependent VHL degradation of HIF1α. We next expressed these constructs in the renal cell carcinoma cell line 786-0 to determine to what extent the VHL-negative phenotype could be reproduced by disruption of HIFα degradation. Matched 786-0 subclones expressing exogenous wild-type VHL (WT) or empty vector (PRC) have been well characterized (Iliopoulos et al., 1995). Of note, 786-0 does not express detectable levels of HIF1α (Maxwell et al., 1999) but does

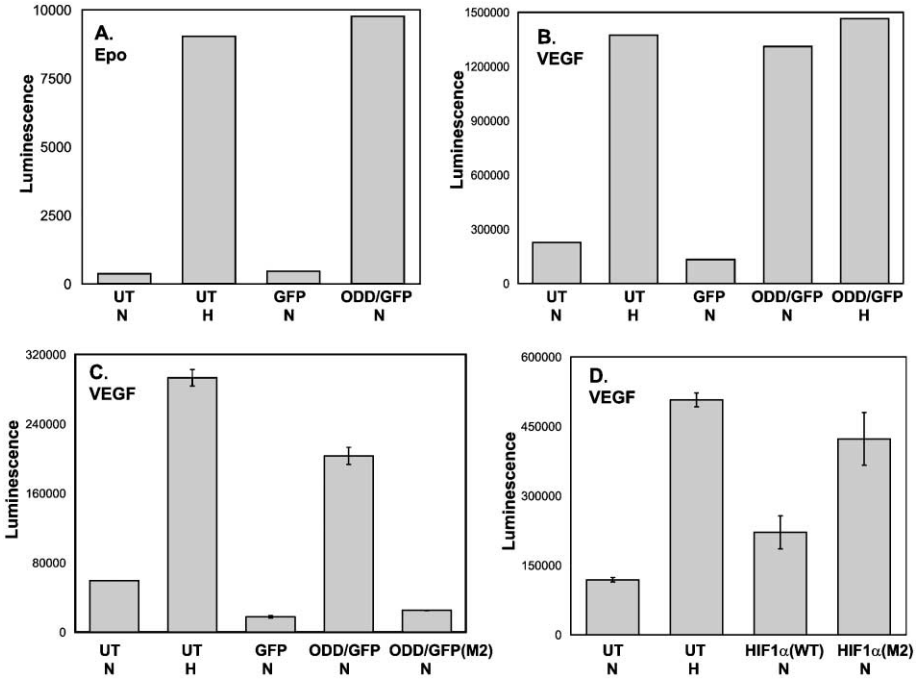

Figure 2. Induction of downstream HIFa transcription activation by ODD-GFP

A: Coexpression of an erythropoetin-luciferase reporter (EPO) activity in Hela cells. Note that ODD-GFP induced transcription levels in normoxia (N) approaching those seen in untransfected cells (UT) exposed to hypoxia (0.5% O_2) for 8 hr (H). **B:** Coexpression of VEGF-luciferase and ODD-GFP. Hypoxic levels of VEGF-luciferase reporter activity were induced by ODD-GFP but not GFP alone. **C:** A 5 amino acid alanine substitution in the ODD (M2) prevented transcription by ODD-GFP. Coexpression of VEGF-luciferase and full length HIF1α cDNA. **D:** Exogenous HIF1α minimally induced VEGF-luciferase. Introduction of the ODD mutation, M2, caused hypoxic levels of reporter activity. Assays were performed in triplicate. Bars represent standard deviation of the mean.

express HIF2α, which appears to be identically regulated by VHL. Stable retroviral lines expressing high levels of ODD-GFP, GFP alone, or HIF1α(M2) were established in both WT and PRC 786-0 backgrounds. Western analysis of whole cell lysates (Figure 3A) confirmed the stabilization of HIF2α in all PRC-derived cells (lanes 1–4) in which VHL was absent. In cell lines that expressed wild-type VHL, HIF2α was only faintly detectable in normoxia (lane 5), consistent with oxygen-dependent ubiquitin-mediated degradation (Maxwell et al., 1999). Addition of GFP alone did not induce endogenous HIF2α levels in normoxia (lane 8). Nor were endogenous HIF2α levels induced by the full-length mutant HIF1α (lane 6), demonstrating that the mutant HIF1α did not interfere with normal VHL function. In constrast, endogenous HIF2α was stabilized in WT cells by the introduction of the competitive inhibitor, ODD-GFP (lane 7), confirming that the ODD derived from HIF1α is capable of blocking VHL binding of HIF2α as well. Western blotting for HIF1α (Figure 3B) confirmed the absence of detectable protein levels in 786-0 cells with or without VHL (lanes 1 and 2). ODD-GFP did not induce endogenous HIF1α expression in 786-0 WT cells (data not shown). HIF1α was detectable only in cells expressing exogenous HIF1α(M2) (lane 3). Downstream transcription activity of HIFα was evaluated in the 786-0-derived cell lines by real-time quantitative RT-PCR for VEGF (Figure 4). VEGF transcription was induced by ODD-GFP (bar 4) and HIF1α(M2) (bar 5), but not by GFP alone (bar 3).

We next studied the effect of HIFα stabilization on another phenotype associated with the presence or absence of VHL. It has been previously demonstrated that in the absence of VHL, 786-0 PRC cells exposed to hepatocyte growth factor/scatter factor (HGF/SF) demonstrate marked branching (Figure 5A).

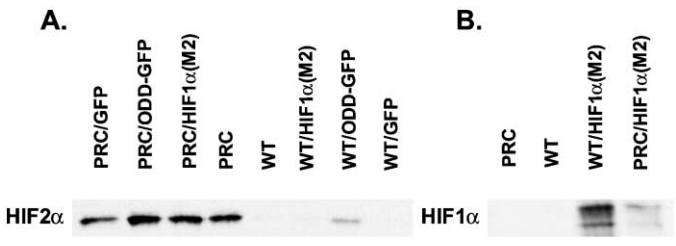

Figure 3. Representative immunoblot of 786-0 stable cell lines with wild-type *VHL* (WT) or empty vector (PRC) expressing ODD-GFP, GFP, or HIF1α(M2)

200 μg lysate per lane. All cells were grown in normoxia except as indicated. **A:** Note that endogenous HIF2α was induced by ODD-GFP, but not by GFP alone or HIF1α(M2). **B:** HIF1α(M2) causes normoxic stable expression of HIF1α.

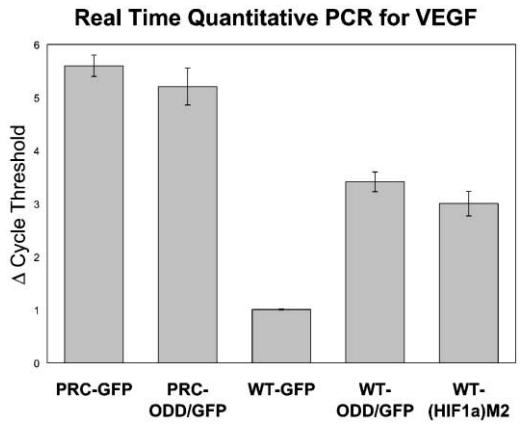

Figure 4. Real-time quantitative RT-PCR for VEGF

Relative cycle thresholds for VEGF amplification. RNA samples were analyzed in triplicate. Standard deviation of the mean for each cell line is indicated.

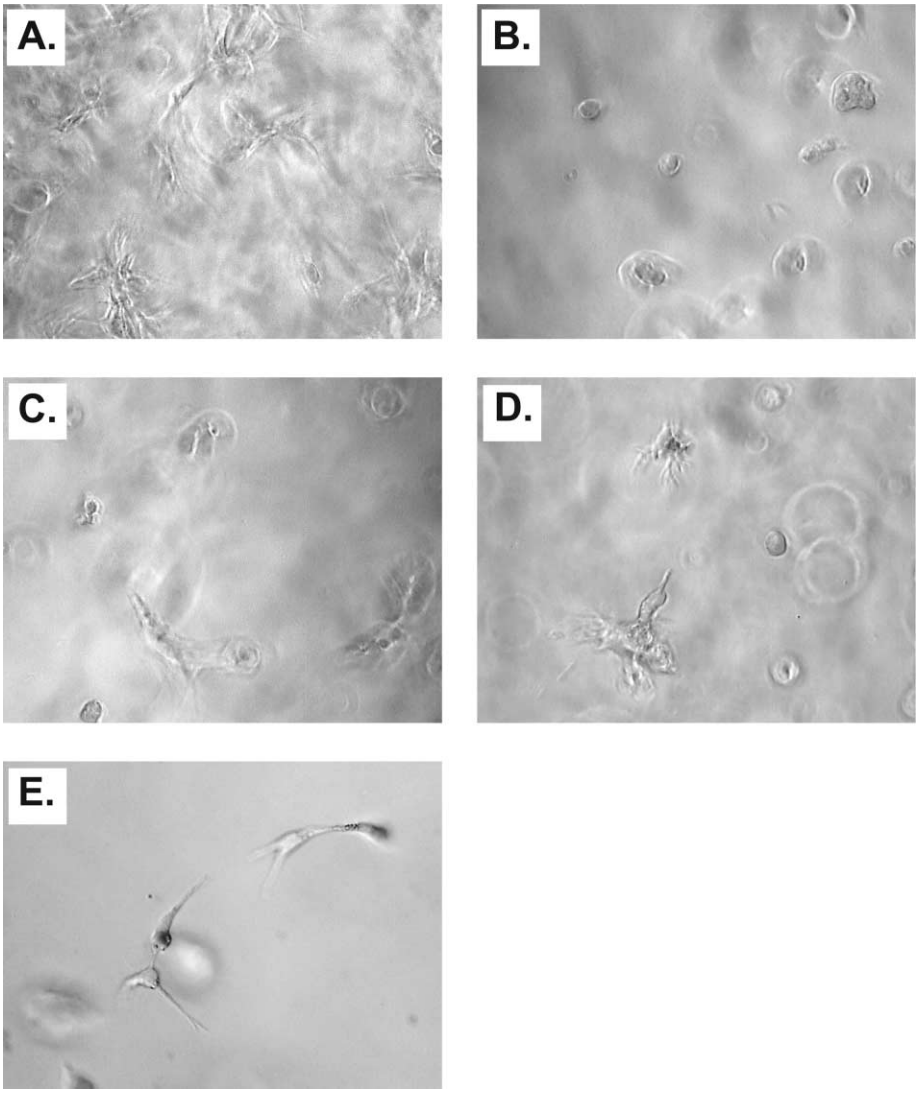

Figure 5. Branching morphogenesis assay

786-0 stable cell lines grown in a colloid matrix under normoxic conditions for 72 hr. **A:** PRC demonstrated branching of all cells. **B:** WT cells expressing GFP only showed no branching. **C:** WT cells expressing ODD-GFP demonstrated a distinct subpopulation of branching cells. **D:** Similarly, WT expressing full-length mutant HIF1α(M2) demonstrated branching of a fraction of cells. **E:** WT cells in hypoxia demonstrate branching in the absence of HGF/SF.

Reintroduction of wild-type VHL completely abrogates this phenotype, resulting in rounded cells (Koochekpour et al., 1999) (Figure 5B). When ODD-GFP was expressed in 786-0 WT, evaluation of three high power fields revealed the branching phenotype in approximately 50% of the cells (Figure 5C). Incomplete branching was attributed to varying levels of expression of the competitive inhibitor, as reflected by the variation of GFP florescent intensity observed between individual cells. These results suggest that the absence of branching in 786-0 cells is dependent on the ability of VHL to recognize its substrates. To test whether the branching observed in PRC cells could result from elevated levels of HIFα, we introduced HIF1α(M2) into WT cells. These cells demonstrated distinct branching morphogenesis in 25%–30% of the cells (Figure 5D), indicating that elevated HIF1α in the presence of wild-type VHL does contribute to the branching phenotype of 786-0 cells. If stabilization of HIFα alone can lead to branching morphogenesis, then we would expect to see the branching phenotype in WT cells exposed to hypoxia. Indeed, hypoxia alone induced clear, though somewhat less vigorous, branching morphogenesis in nearly 100% of these cells (Figure 5E).

Finally, we directly examined the tumorigenic phenotype of VHL deficient cells. 786-0 PRC cells injected subcutaneously into immunocompromised mice form palpable tumors after six weeks. Reintroduction of WT VHL suppresses this tumorigenic potential (Iliopoulos et al., 1995) leading to delayed formation of small tumors after 12–13 weeks. Expression of ODD-GFP abrogated the tumor suppressor effect of VHL in WT cells that were injected into SCID mice (Figure 6A), suggesting that the substrate binding site of VHL is essential for its tumor suppressor function. To determine if the tumorigenicity was due to accumulation of HIF1α, we injected mice with WT cells that expressed HIF1α(M2) (Figure 6B). Elevated levels of transcriptionally active HIF1α in the presence of wild-type VHL did not reproduce the tumorigenic phenotype, suggesting that HIF1α is not the critical target of VHL-mediated ubiquitination for tumorigenesis. Indeed, cells expressing HIF1α(M2) tended toward even lower tumorigenic potential than those expressing wild-type VHL alone. Cells cultured in parallel maintained expression of the mutant HIF1α throughout the duration of the study (data not shown) with no evidence of increased cell death. Histologic examination of the tumors induced by expression of ODD-GFP

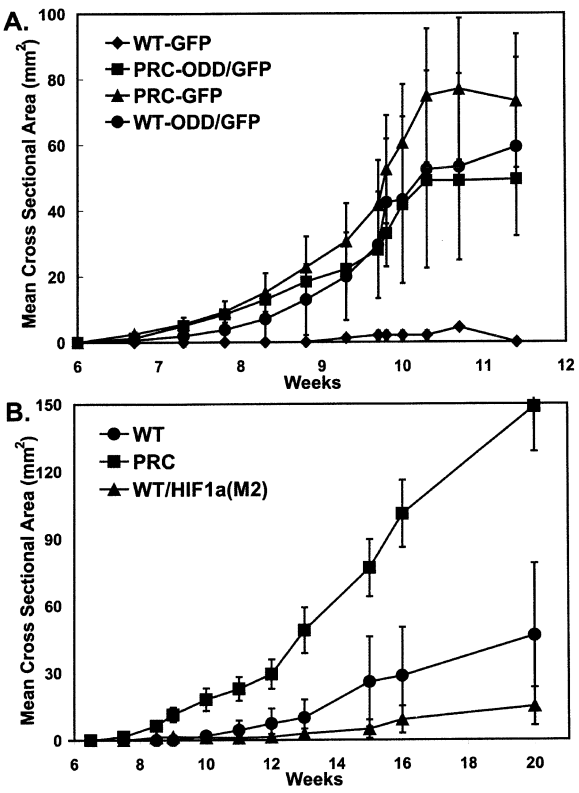

Figure 6. Tumorigenesis assay

SCID mice were subcutaneously injected with 1 million cells as indicated, and tumor kinetics plotted weekly. **A** demonstrates reproduction of the tumorigenic phenotype by the addition of ODD-GFP but not GFP alone. 5 mice were evaluated for each cell line, and mean cross-sectional area determined. Bars represent standard error of the mean at each time point. **B** shows persistent tumor suppression despite overexpression of HIF1α(M2). 7 mice each were evaluated with PRC and WT/HIF1α(M2), and 6 mice were used for the WT line. Means were calculated using all mice in each set, and the standard error of the mean is represented by bars.

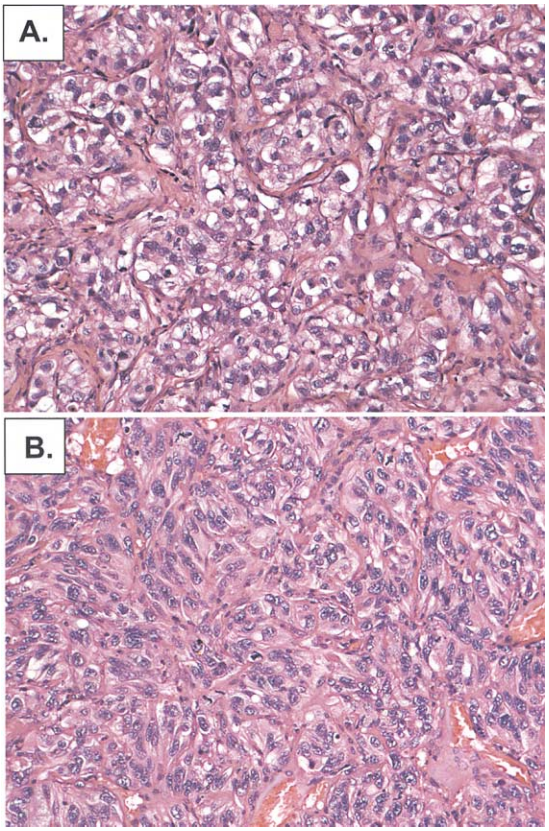

Figure 7. Histologic examination of mouse tumors

A demonstrates the classic clear cell pattern of tumors derived from 786-0 PRC-GFP. **B** shows the poorly differentiated histology uniformly observed for the WT/ODD-GFP tumors. Three tumors from each cell line were sectioned and examined by a pathologist without knowledge of the genotype. The histologic pattern was uniform within each cell line.

(Figure 7) revealed a uniformly poorly differentiated histology, in contrast the classic clear cell pattern of VHL-associated renal cancers. This suggests that loss of some other VHL function, not essential to tumorigenesis and not dependent upon substrate recognition, may be responsible for clear cell histology or, alternatively, that ODD-GFP has other effects in the cell unrelated to its interaction with VHL.

Discussion

The majority of kidney cancers are associated with loss of VHL tumor suppressor function, but the steps leading to tumorigenesis are not fully understood. VHL deficient tumor cell lines demonstrate increased expression of hypoxia inducible genes, but also demonstrate impaired extracellular matrix formation (Ohh et al., 1998) and undergo branching morphogenesis upon stimulation by HGF/SF (Koochekpour et al., 1999). We now know that VHL is the substrate recognition subunit of an E3 ubiquitin ligase that targets HIF1α and HIF2α for degradation. All renal tumor-derived VHL mutations examined result in impaired ubiquitination of HIF1α (Ohh et al., 2000; Cockman et al., 2000). Our objective was to determine to what extent the tumor suppressor function of VHL depends upon its ability to target substrates for ubiquitin-mediated degradation. Using a competitive inhibitor of the VHL-HIF1α binding site, we demonstrate that substrate binding is crucial to tumor suppression by VHL.

Our data show that disruption of VHL-HIFα binding causes accumulation of HIFα protein in normoxia, with corresponding increased transcription of the HIF-dependent genes, VEGF and erythropoietin. This is consistent with the finding that loss of VHL function results in HIFα subunit accumulation in normoxia (Ohh et al., 2000; Cockman et al., 2000), and with the observation that clear cell renal cell tumors demonstrate constitutively elevated levels of VEGF, Glut1, and PDGFα (Siemeister et al., 1996), known targets of HIF. We further show that the branching morphogenesis observed in VHL deficient cells can be reproduced by inhibition of substrate binding. Branching morphogenesis is known to occur upon HGF/SF stimulation through its interaction with the MET receptor (Koochekpour et al., 1999). These results suggest that the branching phenotype of VHL deficient cells may also occur via HIFα stabilization. This is supported by our finding that hypoxia alone can induce branching, even in the absence of HGF/SF. These results are consistent with the recent finding that HIF1α induction occurs as a result of HGF/SF stimulation (Tacchini et al., 2001).

To date, the only two VHL substrates identified have been

HIF1α and HIF2α, α isoforms of the HIF heterodimeric transcription factor (Jain et al., 1998). HIF α is an attractive candidate for a renal cancer oncogene because HIF stabilization results in elevated transcription of a number of angiogenic and proliferative genes, including VEGF (Semenza et al., 1999) and TGF β (Ananth et al., 1999). Indeed, elevated levels of HIF1α have been documented in tumors derived from multiple tissues, including colon, breast, lung, skin, ovary, pancreas, prostate, and kidney (Zhong et al., 1999). Similarly, both HIF1α and HIF2α were found to be elevated in non-small cell carcinoma of the lung (Giatromanolaki et al., 2001), hemangioblastomas, and renal tumors (Krieg et al., 2000).

To determine if overexpression of HIF1α under normoxic conditions can fully explain the tumorigenic phenotype of our cells, we created a mutant of HIF1α that was not able to bind with VHL. Although the possibility that the mutation introduced subtle alterations in transcription complex formation cannot be ruled out, exogenous expression of this mutant did result in normoxic accumulation of HIF1α protein and did increase activation of the VEGF and erythropoietin promoters. Expression of the HIF1α mutant also reproduced branching morphogenesis despite the presence of wild-type VHL. Our results, however, demonstrate that constitutive expression of HIF1-α alone is not sufficient to cause tumorigenesis in 786-0 cells reexpressing wild-type *VHL*. This finding raises several distinct possibilities. It may be that HIF1α and HIF2α have differential tumorigenic potential, and that it is actually HIF2α that is necessary for tumor maintenance in these cells, or a combination of HIF1α and HIF2α. Alternatively, other as yet unidentified VHL substrates may be responsible for the tumorigenic phenotype. With regard to the latter, anti-VHL immunoprecipitates from metabolically labeled cells in the presence of proteasome inhibitors have not revealed any other potential substrates (Maxwell et al., 1999). Also, RNA array results revealed that the majority of VHL regulated genes are also oxygen responsive (Wykoff et al., 2001), again suggesting that the HIFα isoforms are the primary ubiquitination targets of VHL. Finally, we cannot exclude the possibility that our ODD-GFP competitive inhibitor fragment causes tumorigenesis, not through stabilization of HIF α subunits, but by competitive inhibition of normal HIF prolyl hydroxylase activity on as yet unidentified substrates.

We became aware during the course of this study that other investigators were performing a similar set of experiments using the corresponding HIF2α mutant (Kondo et al., 2002 [this issue of *Cancer Cell*]). They report that expression of mutant HIF2α in normoxia does rescue the tumorigenic phenotype in tumor cells transfected with wild-type *VHL*. Thus, it appears that in the context of the multiple genetic changes present in these tumor cells, stabilization of HIF2α is required for maintenance of the tumorigenic state. This is consistent with our finding that the ODD-GFP competitive inhibitor rescued the tumorigenic phenotype, since we demonstrated that the ODD was able to prevent VHL-mediated degradation of endogenous HIF2α in normoxia. We suspect that the 786-0 cell line bears other mutations responsible for tumorigenesis because we find it unlikely that chronic hypoxia alone is sufficient to form tumors.

Despite the fact that both HIF1α and HIF2α are regulated by VHL in normoxia at the protein level (Maxwell et al., 1999), and that both bind to and activate transcription from the same DNA element (Tian et al., 1997), there is data to suggest that they have somewhat different transcription activation profiles. Wiesener et al. reported that HIF2α was a stronger transactivator for VEGF than HIF1α (1998). Similarly, overexpression of VEGF in human hemangioblastomas highly correlated with HIF2α expression in the stromal cells (Flamme et al., 1998). Giatromanolaki et al. also found that HIF2α significantly correlated with VEGF activation and vascularization in lung tumors (2001). Endothelial tyrosine kinase 2 is specifically activated by HIF2α (Tian et al., 1997). It is possible that the enhanced angiogenic profile of HIF2α contributes to a greater tumorigenic potential.

Further, HIF1α and HIF2α demonstrate cell-type specificity with limited redundancy (Jain et al., 1998). HIF2α protein is abundant in vascular epithelium (Tian et al., 1997), particularly in the capillaries of the brain. HIF2α mRNA levels are elevated in lung, liver, and kidney glomeruli, and in the smooth muscle cells of the uterus (Flamme et al., 1997). Interestingly, high HIF2α mRNA levels have also been found in catecholamine-producing cells of the sympathetic nervous system (Favier et al., 1999). Tian et al. reported that mice deficient in HIF2α died in utero due to insufficient circulating catecholamines, despite normal vascular development (1998). This is in contrast to the phenotype of HIF1α knockout mice, which die in utero secondary to neural tube and cardiovascular malformations (Kotch et al., 1999). It is tempting to speculate that if HIF2α is indeed a renal cancer oncogene, then the pattern of HIF2α abundance may be responsible for the observed organ specificity of the VHL syndrome, particularly vascular tumors of the central nervous system and kidney, and catecholamine-producing pheochromocytomas.

In summary, we have shown that competitive inhibition of the VHL substrate recognition site completely abrogates the tumor suppressor function of wild-type VHL protein. Deregulated expression of HIF1α, although capable of inducing the biochemical and morphogenic phenotypes of VHL-deficient cells, is not sufficient to maintain tumorigenesis. These studies do not rule out the possibility that HIF1α could contribute more directly to tumorigenesis in other renal cell carcinomas. It is worth noting that 786-0 cells do not express HIF1α, and that perhaps the ability of specific substrates of VHL to recapitulate the VHL loss phenotype is not an intrinsic property of HIF1α versus HIF2α, but rather is dependent upon the tumor cell in which HIF1α or HIF2α is functioning. The strikingly different effects of deregulated expression of HIF2α and HIF1α on tumorigenicity point to the need to define the distinct downstream targets of these two closely related transcription factors and to determine whether these differences are dependent upon either the lineage or the mutational history of the cells.

Experimental procedures

Cells and cell culture

HeLa and 786-0 cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. 786-0 is a sporadic renal cell carcinoma (RCC) cell line with loss of one *VHL* allele and inactivation of the other allele via truncation of the other after amino acid 104. The sublines 786-0 (wt) and 786-0 (v) were created by stable transfection of wild-type *VHL* or empty vector, respectively, and were a gift from W. Kaelin (Iliopoulos et al., 1995). Transient transfections of HeLa were performed using a 3:1 ratio of Fugene6 (Roche Molecular Biochemicals, Branchburg, NJ) to total plasmid DNA. Cells were grown at 37°C for 24 hr prior to lysis. For the final eight hours of incubation, hypoxic cultures were grown in modular incubator chambers (Billups Rothenburg, Del Ray, CA) containing an atmosphere of 0.5% O_2, 5% CO_2, and 94.5% N_2. Retroviral plasmids, described below, were transfected into the Pheonix 293 viral packaging cell line and incubated

at 32°C for 48 hr. The media was then collected and used to infect 10^5 recipient cells as described. (Kotani et al., 1994) Briefly, the supernatant was filtered and supplemented with 4 μg/ml polybrene (Sigma) and then one milliliter was applied to adherent cells. The plate was centrifuged at 1100 rpm (SorvallRT) for 45 min. A second milliliter of viral media was then added, and centrifugation was repeated. Infected cells were incubated at 32°C overnight and then transferred to 37°C with fresh media. Selection with 1 μg/ml puromycin (Sigma) was started 48 hr later.

Plasmids

pcDNA3-HIF1α, a gift from S. McKnight, contains the HIF1α coding sequence, followed by an in-frame c-*myc* tag, ligated into pcDNA3 (Invitrogen, Carlsbad, CA). To obtain the HIF1α fragment consisting of residues 541 to 580, pcDNA3-HIF1α was PCR amplified with primers 5′-CGACTCCATGGC TGAAGACACAGAA-3′ and 5′-AGTCGGCGGCCGCCAACTGATCGAA-3′ incorporating NcoI and NotI sites, respectively. The product was digested with NcoI and NotI and ligated into pCMV/myc/cyto (Invitrogen) to create an in-frame fusion with c-*myc*. The resulting vector was then PCR amplified with primers 5′-GGACTAAGCTTATGGCTGAAGACACA-3′ and 5′-AGTCGGG ATCCCAGATCCTCTTCTGA-3′, incorporating HindIII and BamHI sites, respectively, to obtain 541–580/c-*myc*. This product was digested with HindIII and BamHI and ligated into pEGFP-N3 (Clontech, Palo Alto, CA) to create an in-frame fusion with enhanced green fluorescent protein (GFP), pEGFP/541–580.

A PCR-based site-directed mutagenesis system (Quikchange, Stratagene, Cedar Creek, TX) was used to replace residues 564–568 of pcDNA3-HIF1α with alanine in two stages. First-stage primers were 5′-GGAGATGT TAGCTCCCTATACCGCAGCGGATGATGACTTCCAG-3′ and 5′-CTGGAAG TCATCATCCGCTGCGGTATAGGGAGCTAACATCTCC-3′, and second-stage primers were 5′-GACTTGGAGATGTTAGCTGCCGCTGCCGCAGC GGATGATGAC-3′ and 5′-GTCATCATCCGCTGCGGCAGCGGCAGCTAA CATCTCCAAGTC-3′. The resulting mutant was PCR amplified with primers 5′-CGACTCCATGGCTGAAGACACAGAA-3′ and 5′-AGTCGGGATCCCAAC TGATCGAAGGA-3′, cut with HindIII and BamHI, and ligated into pEGFP-N3 to create the mutant fusion, pEGFP-541–580(M2).

The high-expression retroviral vector, pVXY, was a gift from Louis Staudt (National Cancer Institute). pEGFP/541–580 was PCR amplified with primers 5′-AGTCGGAATTCATGGCTGAAGACACAG-3′ and 5′-AGTCGCTCGAGT TACTTGTACAGCT-3′, incorporating EcoRI and XhoI, respectively. The product was cut with EcoRI and XhoI and ligated into pVXY to create pVXY/541–580/GFP. The GFP coding sequence from pEGFP-N3 was excised using EcoRI and NotI and ligated into pVXY to create pVXY/GFP.

PEGFP/541–580(M2) was cut with XbaI and blunt-ended using DNA polymerase 1 Klenow fragment. After precipitation, the linearized vector was cut with BamHI and ligated into pVXY between BamHI and a blunted XhoI site to form pVXY/541–580(M2). Retroviral vectors were transformed in Stbl2 competent cells (Life Technologies) and expanded at 30°C.

All PCR was performed using Advantage cDNA polymerase mix (Clontech). All plasmid sequences were confirmed by DNA sequencing analysis. The VEGF-luciferase reporter construct was a gift from Andrew Kung (Bhattacharya et al., 1999) and the erythropoetin-luciferase construct was a gift from Vickram Srinivas (Salceda et al., 1997).

Antibodies

Anti-VHL antibody (Ig32) was from Pharmingen, anti-HIF1α antibody (clone 54) was from Transduction Laboratories (Lexington, KY), anti-GFP antibody (clones 7.1 and 13.1) was from Boehringer Mannheim, and anti-HIF2α was from Novus Biologicals (Littleton, CO).

Immunoblotting and immunoprecipitation

Adherent cells were washed once with phosphate-buffered saline then lysed in 250 μl of 100 mM NaCl, 20 mM Tris-HCL (pH 7.6), Igepal CA630 (Sigma), 5 μM MgCl2, 1 mM sodium orthovanadate, aprotinin (1 μg/ml), "complete" protease inhibitor (Boehringer Mannheim), and 1 mM AEBSF. Lysates were scraped immediately, vortexed, and cleared by centrifugation at 10,000× g at 4°C for 10 min. Protein concentration was determined by the Dc Protein Assay system (Bio-Rad), using bovine serum albumin (Pierce, Rockford, IL) as a standard. For immunoprecipitation, 500 μg lysate was incubated with 5 μg Ab at 4°C for one hour, then 50 μl of protein G-agarose beads (Boeringher Mannheim) were added and incubation was continued at 4°C overnight on a rotator. Beads were washed five times in phosphate-buffered saline with 0.05% Tween 20 (PBST). Lysates were resolved by SDS-PAGE using two-faced gels (8% upper half and 12% lower half) and transferred to PVDF (Immobilon-P; Millipore, Bedford, MA) on a semidry transfer apparatus (Bio-Rad, Hercules, CA). The filter was then incubated for one hour in PBST with 5% powdered milk, washed three times with PBST, and incubated overnight at 4°C in primary antibody (4 μg/ml anti-GFP, 5 μg/ml anti-HIF2α, 1 μg/ml anti-VHL, or 1 μg/ml anti-HIF1α) in PBST with 5% powdered milk. After three more PBST washes, filters were incubated with secondary antibody conjugated to horseradish peroxidase (Amersham) in PBS with 1% powdered milk for 30 min at room temperature. The filter was washed five times with PBST and developed by chemiluminescence (Renaissance; NEN, Boston, MA).

Gene expression assays

In HeLa cells, luciferase reporter activity was measured using a luminescence system (Promega, Madison, WI) as described by the manufacturer. For 786-0 lines, total RNA was extracted with Trizol reagent (Life Technologies, Grand Island, NY) and subjected to real-time quantitative RT-PCR using the TaqMan Gold RT-PCR Kit (PE Biosystems, Alameda, CA) with primer-probe sets designed to amplify Glut-1 and VEGF (Bioserve, Laurel, MD). The VEGF primers were 5′-TACCTCCACCATGCCAAGTG-3′ and 5′-ATGATTCTGCCC TCCTCCTTC-3′ with probe, 5′FAM-TCCCAGGCTGCACCCATGGC-TAMRA-3′. Glut-1 primers were 5′-GCGGAATTCAATGCTGATGAT-3′ and 5′-CAGTTTC GAGAAGCCCATGAG-3′ with probe, 5′FAM-CTGGCCTTCGTGTCCGCCGT-TAMRA-3′. Cycle threshold values were corrected for amplification of a GAPDH control primers-probe (VIC) set (PE Biosystems). Reactions were run on the ABI Prism 7000 Sequence Detection System (PE Biosystems) according to the manufacturer's recommendations.

Branching assays

Assessment of morphogenesis in response to hepatocyte growth factor was performed as described previously (Koochekpour et al., 1999). Briefly, cells were detached by incubation with CellStripper (CellGro) and resuspended in Dulbecco's modified Eagle's medium with 10% fetal calf serum at 350,000 cells/ml. For each flat-bottomed well of a 96-well plate, 60 μl of cells was mixed 1:1 with Matrigel and allowed to set at 37°C for 30 min before adding 120 μl DMEM with 10% FCS and 40 ng recombinant hepatocyte growth factor (R&D Systems). Cells were then incubated at 37°C for 72–90 hr prior to inspection.

Mouse xenograft assay

Approximately 10^6 viable cells, as determined by trypan blue exclusion, were suspended in 100 μl Hank's buffered saline and injected subcutaneously per flank of SCID mice (Taconic). Five to seven 6-week-old female mice were injected with each cell line. Tumors were measured weekly with calipers in the two greatest dimensions by a technician blinded to the genotype.

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